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Animal care and stress paradigm

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Sexually dimorphic neuronal responses to social isolation

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Detailed protocol

Materials and Methods

2.1 Animal Care and Stress Paradigm

All protocols received approval from the University of Calgary Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care guidelines (Protocol # AC13-0027). B6(Cg)-Crh$^{tm1(cre)Zjh}$/J (*Crh-IRES-Cre*)mice and B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-TdTomato)Hze}$/J (*Ai14*) mice, whose generation has been detailed previously(Madisen *et al.*, 2010; Taniguchi *et al.*, 2011), were obtained from Jackson laboratories (stock number 012704 and 007914 respectively). These were maintained as colonies of homozygous mice, with one backcrossing to C57BL/6J background strain following their arrival. Genotyping was used to identify mutants using PCR procedures provided by the supplier. The following primers were used to identify *Crh-IRES-Cre* mutants: *5′-CTT ACA CAT TTC GTC CTA GCC* and *5′- CAA TGT ATC TTA TCA TGT CTG GAT CC-3′* and (468 base pair resultant PCR band). To identify *Ai14* mutants: *5′-GGC ATT AAA GCA GCG TAT CC-3′* and *5′-CTG TTC CTG TAC GGC ATG G -3′* were used (196 base pair band). The age of pre-adolescent mice (post-natal day 21-35) was determined according to previous literature demonstrating that in C57/BL mice the onset of puberty occurs at approximately 5 weeks of age (Nelson *et al.*, 1990; Mayer *et al.*, 2010). Mice were individually housed on a 12h□12h light: dark cycle (lights on at 7:00) with ad libitum access to food and water. Pairs of either homozygous *Crh-IRES-Cre* or *Ai14* genotypes were mated, and the resulting heterozygous *Crh-IRES-Cre;Ai14* offspring used in subsequent experiments. Sixteen hours prior to the acute stress protocol or slice preparation, mice were housed either individually, in same sex pairs or same sex groups (3-5 mice per group). All groups had ad libitum access to food and water. Single or group housed mice were randomly assigned to naive or stress conditions. For stress experiments, mice were exposed to a forced swim stress (between 8:00–9:30 during the light phase) consisting of 20 min in a glass cylinder (14 cm internal diameter) filled with 30–32°C water. Following one hour of recovery in their home cage, mice were anesthetized and brain slices were prepared as described below.

2.2 Slice Preparation

Experimental animals were anaesthetized with isoflurane and decapitated. The brain was quickly removed, and coronal brain slices (250 µm) containing the PVN of the hypothalamus were obtained using a vibrating slicer (Leica, Nussloch, Germany) while submerged in ice cold slicing solution (0 °C, 95% O_2/5% CO_2 saturated), containing (in mM): 87 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 $NaHCO_3$, 25 D-glucose, 1.25 NaH_2PO_4, 75 sucrose. Slices were then allowed a recovery period, of a minimum 60 minutes, in artificial cerebrospinal fluid (aCSF) (32.5°C, 95% O_2/5% CO_2saturated) containing (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose. The CORT synthesis inhibitor, metyrapone was dissolved in polyethylene glycol and injected i.p. 60 minutes prior to swim stress as a dose of 75 mg/kg in a volume of 50 µL. For females isolation experiments, in was dissolved in the drinking water (500 µg/ml) and given for 24 hours prior to isolation and during the entire isolation period.

2.3 Electrophysiology

Hypothalamic slices were transferred to a recording chamber and superfused with 30-32°C aCSF at a flow rate of 1-2 ml/min. Slices were visualized using an upright microscope (BX51WI, Olympus) fitted with infrared differential interference contrast optics. CRH neurons were identified by their expression of tdTomato. Whole-cell patch clamp recordings were obtained from CRH neurons using borosilicate glass microelectrodes with tip resistance between 2-5 MΩ. The normal intracellular solution contained (in mM): 108 K-gluconate, 2 $MgCl_2$, 8 Na-gluconate, 8 KCl, 1 K_2-EGTA, 4 K_2-ATP, and 0.3 Na_3-GTP buffered with 10 mM HEPES. For images of filled cells, 0.2mM Alexa-488 hydrazide and 10 mg·mL^{-1} biocytin was added to internal solution. Microscope images were captured using a Retiga EXi camera (Qimaging) and processed using ImageJ.

Recordings were amplified using a Multiclamp 700B amplifier (Molecular Devices, Union City, CA), low-pass filtered at 1 kHz, and digitized at 10 kHz using the Digidata 1322 (Molecular Devices). Data were recorded (pClamp 9.2; Molecular Devices) and stored on a computer for offline analysis. During all experiments initial access resistance (R_a) was below 20 MΩ, membrane capacitance (C_m) was above 300 MΩ. Cell membrane properties were monitored for the duration of the experiments and only recordings in which changes to R_a C_m did not exceed 15% were accepted for analysis. For synaptic experiments, the cell membrane was voltage clamped at -80mV. Spontaneous inhibitory ionotropic $GABA_A$ receptor postsynaptic currents (sIPSCs) were isolated by blocking AMPA- and kainate-receptor-mediated glutamatergic synaptic transmission with 6,7-dinitroquinoxaline-2,3-dione (DNQX ,10 µM). When measuring spontaneous excitatory postsynaptic currents (EPSCs), the $GABA_A$ channel blocker picrotoxin (100 µM) was

included in the bath to isolate excitatory currents mediated by AMPA and kainate receptors. These spontaneous currents representing stochastic transmitter release were analyzed using MiniAnalysis 6.0.3 (Synaptosoft, Decatur, GA). Event detection was set at three times the baseline noise and confirmed as synaptic events by eye.

To determine RMP, cells were recorded in zero current (I=0) mode. Latency to spike initiation and firing thresholds were both measured using a current clamp depolarization step protocols. First, a baseline current injection that maintained the membrane voltage near -80mV was chosen individually for each cell, which served to exclude the possible confounding influence of variable resting membrane potentials between cells. Next, a 200ms/30pA hyperpolarizing current injection was given followed by 250ms/20pA depolarizing current steps up to 140pA. The latency time was measured as the duration from the point of initiating the depolarizing pulse to the initiation of the first spike. Firing threshold was considered the membrane potential at the initiation of the first spike. Both firing threshold and membrane potential are corrected for a liquid junction potential of 12mV, as calculated with solution ion concentrations.

In experiments examining cell properties following 4-AP administration, baseline recordings were obtained, and then a ten-minute treatment period was allowed before secondary data were obtained. Synaptic currents were evoked by paired afferent stimulation (every 5s with an interstimulus interval of 50ms) and analyzed using Clampfit 9.2 (Molecular Devices). Evoked postsynaptic current (ePSC) amplitudes are calculated from the baseline (current before the first evoked response) to peak of each evoked response. The paired pulse ratio (PPR) was calculated using the ratio of the amplitudes of the evoked pair (peak 2/peak 1) from a minimum of a one-minute epoch within each cell. To isolate the voltage gated fast inactivating potassium current the following cocktail was applied to the slices for at least of 15 min prior recording: Bicuculline 10 µM, DNQX 10 µM, dAPV 50 µM, TTX 1 µM, TEA 20 mM.

2.6 Data Analysis

Each group represents a minimum of three animals for pharmacology experiments, or a minimum of four for stress experiments. Data points are presented as mean ± SEM. Statistical analyses for sIPSCs, and current clamp step data were performed in GraphPad Prism 4 using a one way ANOVA for multiple groups followed by a post-hoc Tukey's multiple comparisons test; unpaired student's t-test were used for two group comparisons, and a K-S statistic for comparing two distributions.

2.7 Drugs

Drugs were dissolved into aCSF daily prior to experiments from frozen aliquots stored at -20°C and added to the bath by perfusion pump. The drugs were dissolved in accordance with guidelines either in DMSO, PEG, ethanol or distilled water. DNQX, 4-AP, d APV and metyrapone were obtained from Tocris (Tocris Cookson, Ellisville, MO). picrotoxin, bicuculline, TEA and corticosterone were obtained from Sigma (Sigma- Aldrich, St. Louis, MO), TTX from Alomone labs (Jerusalem BioPark (JBP), Hadassah Ein Kerem, P.O.Box 4287 Jerusalem 9104201, Israel).

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1. Bains, J. (2020). Animal care and stress paradigm. Bio-protocol Preprint. bio-protocol.org/prep571.
2. Senst, L., Baimoukhametova, D., Sterley, T. and Bains, J. S.(2016). Sexually dimorphic neuronal responses to social isolation. eLIFE. DOI: 10.7554/eLife.18726